Endothelial cell transfections: a way for studying promoter activity of genes involved in atherogenesis

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Premises and terminology

By gene therapy we call the entire array of strategies by which a nucleic acid, usually DNA, is administered with the aim of modifying the genetic repertoire of target cells[1]. The aim of such an approach is to change the phenotypic response of the target cell, either through the synthesis of the gene product (the protein) encoded by the newly introduced nucleic acid, or through a change in the expression of a target gene. The former strategy utilizes the transcription and translation of the gene introduced, and thus exploits the cellular transcription and translation machinery allowing the production, from one single gene, of several copies of messenger RNA (mRNA), and from each of these several copies of the encoded protein. With the latter strategy, we aim at an interference with the mechanisms of transcription and translation. While the latter strategy may use, according to the case, either DNA or RNA, the nucleic acid is always DNA in the former.

In order to address a specific molecular target *in vivo* with techniques of gene transfer, two main ingredients are necessary, both more or less simultaneously developed in these last two decades. The first ingredient has been the development of recombinant DNA technology, which has already yielded important therapeutic applications (see the production of tissue plasminogen activator, factor VII, and human insulin). In these cases present technology is adapted to the production, by bacteria or eukaryotic cells, of large amounts of proteins normally not produced by cells used for this purpose. Such techniques have been subsequently adapted to *in vivo* or *ex vivo* gene transfer in humans. The second ingredient, yet to be much improved, is the mastering of molecular mechanisms at the basis of pathological processes. Our imperfect knowledge of the complex mechanisms involving multiple genes at the basis of atherosclerotic vascular disease has so far determined a largely empirical approach to the use of gene therapy techniques in vascular disease. In other words, we have a potent gun, but we do not know yet where to aim precisely. Techniques of gene transfer into cells indeed also promise to deepen our knowledge of pathogenic mechanisms of disease, through an improved analysis of mechanisms of regulation of gene expression. Some examples of these uses will briefly illustrate such possibilities.

How to alter the genetic repertoire or gene expression of target cells to change vascular responses: basic concepts

To ensure the transcription of a foreign gene into mRNA and its subsequent translation into protein, this has first to be inserted in a plasmid, i.e. a circular double-helix DNA molecule capable of self-replicating in an eukaryotic host. The process through which exogenous sequences of a nucleic acid are introduced into a cell is named transfection . We will first review the constitutive characteristics of a nucleic acid

suitable for this purpose, and then how transfections in vascular cells are performed. These concepts are the basis to understand practical options nowadays available, and directions now taken for vascular gene therapy.

Construction of the nucleic acid. In the setting up firstly *in vitro* of methods for eukaryotic gene transfections, it is necessary to clearly distinguish the product of transcription of the transfected construct from normal constitutive or induced gene products of the target cell. In order to control the success of such procedures, several prokaryotic genes are used, whose products, easily detected and measured, do target cells never normally transcribing them. These genes are therefore markers (reporters) of the transfection occurred. An example for a largely used reporter gene is that of chloramphenicol acetyl transferase (CAT). CAT is an exclusively bacterial protein, produced by some *Escherichia coli* strains when they are exposed to the antibiotic chloramphenicol. The expression of CAT promotes the acetylation and, consequently, the inactivation of chloramphenicol, thus conferring antibiotic resistance to the bacterial strain. The detection of CAT activity (as well as of beta-galactosidase activity or of the activity of other reporter genes) allows us to measure the transfer of foreign genetic material normally not present in the repertoire of an eukaryotic cell.

In order to have the expression of the new protein it is however also necessary that in the plasmid, in addition to the gene, some of its regulatory sequences, named promoters and enhancers, are also inserted[2]. As can be seen in figure 1, the gene transfer, within a plasmid, of the sole CAT reporter gene does not *per se* lead to any expression of the protein. On the contrary, this is transcribed in large amounts when the CAT gene is bound to a viral promoter, for example that of the SV40 virus. It is indeed the non-coding region of the construct, containing the strong viral promoter, to allow, once the construct itself has been delivered inside the cell, the rapid transcription of the gene downstream the promoter (Fig. 1)[3]. In order to produce, first *in vitro* and subsequently *in vivo*, a transfectable gene construct it is therefore necessary to bind the gene of interest to a promoter. This principle has been followed in the development of techniques of human gene therapy, which have been a logical extension of the *in vitro* approaches introduced in research laboratories little more than 15 years ago.

The choice of cells to transfect and of modes for transfection. For an effective gene transfer into cells of a living organism, two alternative approaches are available, with consequences on the precision of transfection of the target cells[4,5]. A first type of approach is to aim only at the cell identified as the target. This is done, for example, in gene therapy for cystic fibrosis, attempting to restore the defective gene of the chloride transporter in cells of the respiratory mucosa. Another example is

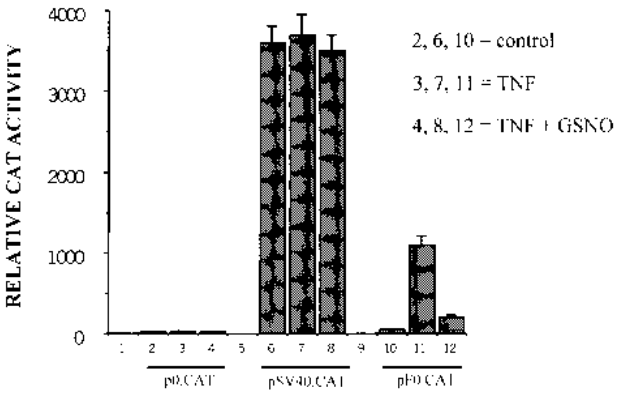

Figure 1. *Transfection studies in bovine aortic endothelial cells using promoterless gene constructs (p0.chloramphenicol acetyl transferase-CAT), highly expressed constructs containing the strong SV40 viral promoter, and constructs with a physiological promoter for the vascular cell adhesion molecule-1 (VCAM-1). The insertion of the above plasmids into endothelial cells was obtained in these cases with the calcium phosphate coprecipitation technique (see text). Notice that gene expression, here measured through the activity of the reporter gene for CAT, is absent when the CAT gene is bound to the strong viral promoter (SV40.CAT), but in this case not subject to controls by cytokines [such as tumor necrosis factor (TNF)-α, used in these experiments, or nitric oxid e (NO)]. Conversely, the insertion of the physiological VCAM-1 promoter upstream of the reporter gene confers the physiological regulability to the VCAM-1 gene, consisting of the inducibility by TNF-α and the negative regulation of such induction by NO. When we intend, with gene therapy approaches, to overexpress a given gene, this is bound to a strong viral promoter, similar to the one here used. Such an approach has been for instance followed in the construction of the plasmid for vascular endothelial growth factor used for the first attempts of vascular gene therapy in humans. GSNO = NO donor S-nitroso-glutathione. From De Caterina et al.[3], modified.*

gene therapy for some cancers, introducing the gene for a tyrosine kinase, which is able to convert the pro-drug gancyclovir into an active toxic product inducing cell death. Such treatments are, by choice, exclusively directed to the transfected cell.

An alternative approach may be however the insertion of a cell in a gene whose product is then able to influence, with a paracrine mechanism, neighboring cells. This has been done, for example, in attempts to promote coronary angiogenesis, or in those aiming at increasing the production of hormonal products.

Another important distinction is between approaches of gene therapy requiring the isolation of target cells, the *in vitro* gene transfer in such isolated cells, and the subsequent reintroduction of engineered cells in the patient s organism (*ex vivo* gene therapy) and approaches by which transfection is directly attempted *in vivo*. For organs or organ segments that are difficult to access for sampling, it is indispensable to rely on these techniques allowing the direct penetration of the gene into the target tissue.

Methods of gene transfer in vascular cells and myocytes. To obtain the transfer of foreign genetic material within cells, several methods have been developed, grouped in three big categories[4,6,7] (Table I). These include: a) physical and chemical methods (naked DNA and cationic liposomes); b) methods based on the use of viral vectors, therefore producing real local infections (retroviruses, adenoviruses, adeno-associated viruses);

Table I. Vehicles and vectors for gene transfer.

Vehicle/vector	Advantages	Disadvantages
Plasmids-liposomes	Non immunogenic Easy to produce No limitations in DNA dimensions	Low efficiency No integration into host genome
DNA-protein complexes	Absence of vital viruses No limitations in DNA dimensions	Efficiency *in vivo* unknown Duration of expression unknown
Viral fusigenic liposomes (liposomes conjugated with proteins of hemagglutinating virus of Japan (Sendai virus)	Absence of vital viruses No limitations in DNA dimensions High efficiency	Safety still unknown Possible immunogenicity
Retroviruses	Well known Long duration of expression Integration into host genome	Only work replicating cells Transgene has to be < 8 kb Low viral titer (< 10^7/ml) Associated with risk of lymphoma in monkeys
Adenoviruses	Only work on differentiated cells High *in vivo* efficiency High viral titer (10^{10}/ml)	No integration into host genome Complex viral structure Potentially pathogenic Strong immune reaction Short duration of expression Transgene has to be < 8 kb May recombine with wild adenoviral strains
Adeno-associated viruses	High *in vivo* efficiency	Not yet used in vascular gene therapy Potential contamination with adenoviruses Possible integration into host genome
Herpes viruses	High *in vivo* efficiency	Not yet used in vascular gene therapy Potentially pathogenic

and c) conjugated viral vectors (adenovirus-augmented receptor-mediated vectors, hemagglutinating virus of Japan, Sendai virus-liposomes)[8].

The first evidence for the practical possibility of transfecting foreign genetic material within mammalian cells was obtained through the use of physic and chemical methods, such as the so-called calcium phosphate co-precipitation [9]. This technique requires the suspension of the plasmid in a calcium phosphate solution, which is then applied on cultured cells. The ensuing precipitation of the salt and the formation of salt crystals on the cell surface facilitate the transfer of genetic material through plasma membrane (Fig. 1). This system however allows the transfection of few cells, in a number variable between 1/1000 and 1/10 000. The term transfection efficiency indicates the percent of cells exposed to the transgene that show evidence of expression of the transgene. This varies, in this case, between 0.1 and 0.001%. Among other physical and chemical vehicles, the so-called cationic liposomes are also of interest. These are lipid vesicles containing the solution with the plasmid to transfect, which allow the fusion with plasma membrane and the passage of the liposomal content into the cytoplasm. The cell, through a process of micropinocytosis, is thus able to internalize the microsomal particles; the nucleic acid sequence is thus also partially protected from lysosomal degradation. For their cationic charge, liposomes form electrostatic bonds with DNA molecules[10,11].

Methods of gene transfer based on the use of viral vectors exploit the intrinsic capability of viruses of transferring genetic material into infected cells. Some viruses (retroviruses) are able to insert (integrate) their genetic material into the genome of the host cells, while others maintain their genome in the nucleus separated from that of the infected cell. Viral vectors of both types have been produced and used for gene therapy.

Theoretically, an ideal method of gene transfer should be able to transfer an adequate amount of genetic material for a sufficiently long time into a relevant population of target cells, in order to reach the desired therapeutic effect without accessory risks for the patient. In practice, this is still, at the present time, impossible to realize, and there are only variable approximations to the ideal vector. Each of the methods of gene transfer available has some limitations, but it is interesting that the choice of systems available can now be done based on the objective to reach (Table I). Physical and chemical methods for gene transfer (naked DNA and liposomes) have the advantage of being practical, easy to prepare, and safer as to side effects, but are, as discussed above, much less efficient than viruses in transferring genes to cells and maintaining them active over time. On the other hand, viral vectors are able to transfer the gene of interest with greater efficiency, but have problems of susceptibility to specific destruction by the host immune system. In addition, retroviral vectors, which allow us to obtain

a stable genomic insertion of the transfected material because of its chromosomal incorporation, expose the target cell to the risk of insertion mutations. Moreover, a state of cell proliferation is necessary. This characteristic is compatible, for example, with the use of these vectors for the transfection of concanavalin A-stimulated T lymphocytes, as has been done in the defects of adenosine-deaminase in the syndrome of combined immune deficiency[12]. In the cardiovascular area, this approach has been used in processes of restenosis partially caused by smooth muscle cell proliferation[13]. Retroviral vectors have little chances of use in the case of slow-proliferating cells, such as endothelial cells. Finally, adenoviral vectors, at variance from retroviruses, do not require proliferating cells for efficient gene transfer[14], but induce a transient transfection, lasting a few weeks, and may cause a state of local inflammation[15-17]. Some of these disadvantages may be overcome by adeno-associated viruses[18].

It is however possible to choose the method of most suitable gene transfer according to the effect which we want to obtain. As an example, while the expression of proteins which have to remain within the cell requires a high transfection efficiency, presently only offered by viral vectors, this is not required in the case of proteins which will have to be secreted and only act with paracrine effect. In this case, there is no need for a high transfection efficiency and non-viral methods are therefore suitable. Such considerations have guided most recent successfully used techniques in vascular gene therapy.

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